

Food Restriction Markedly Increases Dopamine D2 Receptor (D2R) in a Rat Model of Obesity as Assessed With In-Vivo μ PET Imaging ($[^{11}\text{C}]$ Raclopride) and In-Vitro ($[^3\text{H}]$ Spiperone) Autoradiography

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ABSTRACT **Introduction:** Dopamine (DA) regulates food intake by modulating food reward and motivation but its involvement in obesity is much less understood. Recent evidence points to the involvement of leptin in the DA-related modulation of food intake. Here we assess DA D2 receptors (D2R) in a genetic rodent obesity model characterized by leptin-receptor deficiency and assess the influence of food restriction on these receptors. **Methods:** We compared D2R levels between Zucker Obese (fa/fa) and Lean (Fa/Fa) rats at 1 and 4 months of age and in two different feeding conditions (restricted and unrestricted food access) using in-vivo μ PET imaging ($[^{11}\text{C}]$ raclopride, which is a method sensitive to competition with endogenous DA) and in-vitro ($[^3\text{H}]$ spiperone washed to ensure no competition with endogenous DA) autoradiography (ARG). **Results:** Both ARG and μ PET showed that D2R were higher at 1 month than at 4 months of age and that food restricted animals had higher D2R than unrestricted animals. However there were significant differences in the results obtained at 4 months between ARG and μ PET. ARG showed that at 1 month and at 4 months unrestricted lean rats (Le U) had significantly higher D2R binding than obese unrestricted rats (Ob U) but showed no differences between restricted obese (Ob R) and restricted lean rats (Le R). It also showed that D2R decline between 1 and 4 months of age was significantly attenuated in food restricted rats [both obese and lean]. In contrast, μ PET showed that at 4 months of age, Ob U showed greater D2R availability than Le U rats but like ARG showed no differences between Ob R and Le R rats. **Conclusion:** The lower D2R binding in Ob U than Le U rats observed with ARG most likely reflects decreases in striatal D2 receptors levels whereas the increased availability observed with μ PET is likely to reflect reduced DA release (resulting in decreased competition with endogenous DA). Lack of a significant difference between Ob R and Le R suggests that the differences in dopamine activity and D2R levels between Ob and Le Zucker rats are modulated by access to food. The ARG finding of an attenuation of the age-related loss of D2R binding corroborates previous studies of the salutary effects of food restriction in the aging process. Because $[^{11}\text{C}]$ raclopride is sensitive to competition with endogenous DA, the higher D2R binding in obese rats with raclopride despite the lower D2R levels shown with spiperone could reflect lower extracellular DA in the Ob rats and merits further investigation. **Synapse 62:50–61, 2008.** Published 2007 Wiley-Liss, Inc.[†]

INTRODUCTION

Obesity is one of the major and fastest growing public health problems worldwide (Flegal and Ogden 2002). The increases in obesity are likely to reflect

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the interplay between susceptibility genes in a changing environment where food is widely available, diverse, and highly palatable (Friedman, 2004). Food intake is regulated not only by nutrient and caloric requirements but also by its reinforcing properties (Carr, 2006). Dopamine (DA) is one of the neurotransmitters modulating the reinforcing properties of food (Carr, 2006). Indeed DA cells fire (increasing DA release in the nucleus accumbens) when expecting food and their firing in turn is influenced by food deprivation (Meguid et al., 2000). While DA has been classically described as a neurotransmitter involved with locomotor activity and reward, more recent work has shown DA to be associated with the prediction of reward (Phillips et al., 2007; Schultz et al., 2000), with the motivation to procure the reward (McClure et al., 2003; Phillips et al., 2007) and with the facilitation of conditioned learning (Fenu and Di Chiara, 2003). PET imaging studies in humans have recently corroborated an involvement of DA both in food reward (Small et al., 2003) and in conditioning to food cues (Volkow et al., 2002).

The involvement of DA in overeating and obesity has been supported both by preclinical and clinical studies (Hamdi et al., 1992; Meguid et al., 2000; Wang et al., 2001, 2003). Studies done in animal models of obesity (*ob/ob* mice, obese Zucker rats, obesity-prone Sprague–Dawley rats and seasonally obese animals) have documented reduced DA activity in the tuberoinfundibular pathway that projects to the hypothalamus (Friedman, 2004). In these animal models, treatment with DA agonists reverses the obesity; presumably by activation of D2R and D1R (Pijl, 2003). Evidence of DA's involvement in obesity, particularly through its interactions with D2R, is given by the higher risk of weight gain and obesity observed in patients chronically treated with antipsychotic drugs (D2R antagonists) (Friedman, 2004). Also PET brain imaging studies have documented reductions in D2R availability in the striatum of obese individuals (Wang et al., 2001). In the obese subjects but not in the controls, D2R availability levels were inversely related to the body mass index (BMI), suggesting an involvement of the DA system in excessive weight gain. In contrast, PET studies done in patients with anorexia nervosa have reported the opposite; higher than normal striatal D2R availability (Frank et al., 2005).

The Zucker rat is a popular model of obesity in which a mutation in the leptin receptor (Chua et al., 1996) causes obesity, diabetes, and hyperphagia (Fetissov et al., 2000). Zucker rats with this mutation are obese while their littermates without the mutation are lean. The hormone leptin, which is a key modulator of food intake and energy balance (Hagan et al., 1999) has been shown to also modify DA cell activity (Krugel et al., 2003). Moreover, leptin receptors are

extensively expressed in DA neurons of the VTA and SNc (Figlewicz et al., 2003) suggesting that leptin may modulate feeding behaviors in part via its effects on dopaminergic neurotransmission. Here we utilized the obese (*fa/fa*) Zucker rat to investigate the involvement of DA in the obese phenotype under conditions of restricted and unrestricted food access. We hypothesized that obese rats (leptin deficient) given chronic unrestricted access to food would have lower D2R levels and decreased DA release than their lean counterparts. We also hypothesized that chronic food restriction would attenuate such differences.

MATERIALS AND METHODS

Animals

Male 1 month old Zucker Obese (*fa/fa*) (Ob; $N = 20$; and Lean (*Fa/Fa*) or (*Fa/fa*) (Le; $N = 20$) rats were used. Rats were divided into four groups of 10 rats in each group. Specifically, (1) Ob rats with unrestricted (U) food access, (2) Ob rats with restricted (R) food access, (3) Le U rats and (4) Le R rats. At 1 month of age half the rats were placed on restricted food access (70% of ad-libitum fed animals) and the rest on free access to food (ad-libitum). Rats were obtained from Harlan (Indianapolis, IN) and housed on a reverse 12 h light/dark cycle with lights off at 7 am. All experiments were conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (NAS and NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

Behavior assessment—Food intake and weight

Rats were fed a standard (Purina) laboratory rat chow. Food intake was monitored daily at 1500 h and all rats were weighed every other day. Restricted diet rats were fed daily at 1500 h and the amount of food given was continuously adjusted to 70% of food intake of the ad-lib rats.

Behavior assessment: Effects of apomorphine

Rats were placed in an Optical Sensor Plexiglas arena (Minimitter Corporation, Oregon, USA) which consisted of an optical sensor that was securely attached to the wire top of the animal home cage. After an initial one-time 60 min habituation session, each animal was given two 60-min sessions in the same boxes: (1) saline and (2) apomorphine (APO) (1 mg/kg) on two consecutive days. Saline injections always preceded APO injections. Optical beam breaks were recorded every minute and the total number of beam breaks for each group of animals was summed into 60-min sessions.

Experiment 1: in-vitro [^3H] spiperone autoradiography

D2R autoradiography

Each animal was deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The brain was rapidly removed and frozen in an isopentane and dry ice bath and stored in a -80°C freezer. The brain was then transferred to a cryostat (Leica CM3050s), and sections were cut 14 μm thick at -18°C . Sections were mounted on glass microscope slides and stored at 80°C until binding was conducted. Slides were gradually brought back to room temperature and then preincubated at room temperature for 10 min in 50 mM Tris-HCl buffer (pH 7.4). The slides were then incubated in specific binding buffer (50 mM Tris-HCl, 0.4 nM [^3H] spiperone, 10 μM ketanserin tartrate) at room temperature for 1 h. To determine nonspecific binding some slides in parallel were incubated for 1 h in specific binding buffer in the presence of 10 μM haloperidol. Next, the slides were washed 2×10 min in ice-cold 50 mM Tris-HCl and ice-cold dH_2O . Afterwards, 20 μl aliquots of incubation solution were taken and added with 3 ml of Ultima Gold XR (scintillation solution) and the amount of radioactivity was measured with a liquid scintillation counter. Slides were then dried overnight in a dessicator and placed in a glass slide cassette for qualitative and quantitative analysis using a β -Imager (Biospace, Paris, France). Using Betavision software (Biospace, Paris, France/Capintec, USA), Regions of Interest (ROI's) were drawn on the left and right striatum (ST), cerebellum (CB), cingulate (CG), and frontal cortices (FC) of each brain slice. All regions were identified by comparing the radioactive images to templates from the Paxinos and Watson rat atlas (Paxinos and Watson, 1986). The data was then calibrated using a tissue homogenate standard. Mean values ($\mu\text{Ci/g}$) of the left and right receptor rich regions (ST, CG, and FC) for each group were later normalized for receptor poor regions (CB) and the data are reported in respective ratios (receptor rich/receptor poor).

Experiment 2: in-vivo [^{11}C] raclopride D2r μPET MRI imaging

For accurate qualitative and quantitative image analysis using μMRI - μPET image coregistration, MR templates from 1 and 4 month old lean Zucker rats were acquired on a 4T Siemens scanner using the 2d proton density sequence with the following optimized parameters: TR/TE 200/20 ms, a 256×256 matrix, a field of view of 32×32 mm and a slice thickness of 1 mm with an in plane spatial resolution 194 mm.

D2R μPET imaging

μPET assessment of D2R in these rats was performed using a μPET R4 Scanner (Concorde Micro-

systems). Each animal was fasted for 24 h before being scanned in the μPET . Next each rat was anesthetized and injected intravenously with [^{11}C] raclopride, a DA D2R-specific ligand, and dynamic PET scanning followed for 60 min. Raclopride was purchased from Sigma and [^{11}C] raclopride was synthesized as described previously (Volkow et al., 1999).

Rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic head holder (David Kopf Instruments; CA) in a prone position on the bed of the scanner. Next, the lateral tail vein was catheterized and 250 μl of blood were collected from the rats in EDTA_K coated Multivette (Sarstedt, USA) tubes prior to [^{11}C]raclopride administration. Blood samples were centrifuged at 3000 rpm for 10 min; plasma was collected and immediately placed on dry ice.

Animals were then injected via the same tail vein catheter with [^{11}C] raclopride ($424 \pm 49 \mu\text{Ci}$); specific activity, 1.7–12.3 mCi/nmol). Injected volumes were $\sim 400 \mu\text{l}$. Total acquisition time was 60 min (24 frames: six frames, 10 s; three frames, 20 s; eight frames, 60 s; four frames, 300 s; three frames, 600 s), and data were acquired in fully three-dimensional mode with maximum axial acceptance angle ($\pm 28^\circ$). Images were constructed using Fourier rebinning (Matej et al., 1998) followed by two-dimensional filtered back-projection with a ramp filter cutoff at Nyquist frequency.

To control for the effect of 24 h fasting of unrestricted rats we scanned these rats two times separated by 2 weeks. One scan consisted of unrestricted rats without any fasting (food always available right up to scan) and the second scan consisted of the same rats with 24 h fasting prior to scan.

μPET analysis

Using the Pixel-Wise Modeling Software Suite (PMOD Technologies, Switzerland), μPET images were co-registered with the previously generated MRI templates. Initially the two images were co-registered using the method of Mutual Information (MI) (Woods et al., 1992) (Woods et al., 1993) through a MI algorithm implemented within the PMOD environment, followed by a manual adjustment of the μPET image in all three planes (sagittal, coronal, transversal) so that the Harderian glands and caudate putamen from both imaging modalities matched. ROI's for left and right ST and CB were selected using the μMRI - μPET coregistered Harderian glands as a reference point. Specifically the ST and CB for each animal were identified as eight and 12 slices respectively, caudal to the Harderian glands (slice thickness, 1.2 mm). It has been shown previously that the Harderian glands (located just rostral of the brain), because of their uptake of radioactivity, are routinely used as markers

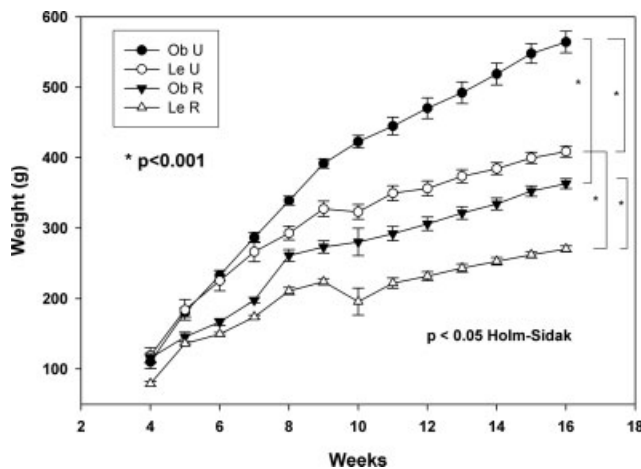


Fig. 1. Mean (\pm SEM) weight over development across groups. (*) $P < 0.05$.

in rodent PET studies (Fukuyama et al., 1998; Hume et al., 1996; Kuge et al., 1997).

Qualitative assessment of μ PET images was performed, and representative μ PET images of each rat were acquired. The image analysis was performed using the Fusion, PxMOD and Kinetic programs included in the PMOD v. 2.65 software suite. Quantitative analysis of the μ PET images consisted of the MRTM0 Ichise Binding Potential (BP). This multireference tissue model provides an accurate noninvasive method of estimating D2R binding potential without having to sample blood (Ichise et al., 2003).

Plasma insulin and leptin concentrations

Plasma insulin and leptin concentrations were measured in duplicate with commercially available insulin and leptin enzyme immunoassay (EIA) kits obtained from Linco Research (Linco Research, Missouri, USA). Samples were taken 24 h after last meal.

RESULTS

Body weight

A multifactor three-way ANOVA revealed significant main effects in body weight with respect to Strain ($F = 461.683$; $df = 1, 79$; $P < 0.001$, Fig. 1), Diet ($F = 320.027$; $df = 1, 79$; $P < 0.001$, Fig. 1), and Age ($F = 5288.733$; $df = 1, 79$; $P < 0.001$; Fig. 1). At 1 month, there were no differences between groups. At 4 months, Ob U rats showed higher weight gain than Le R ($t = 23.873$; $P < 0.05$), Le U ($t = 26.545$; $P < 0.05$), and Ob R ($t = 28.154$; $P < 0.05$). Similarly, at 4 months, Le U and Ob R showed significantly higher weight gain compared to Le R, ($t = 7.441$; $P < 0.05$), and ($t = 5.832$; $P < 0.05$), respectively. There were no differences between Ob R and Le U rats.

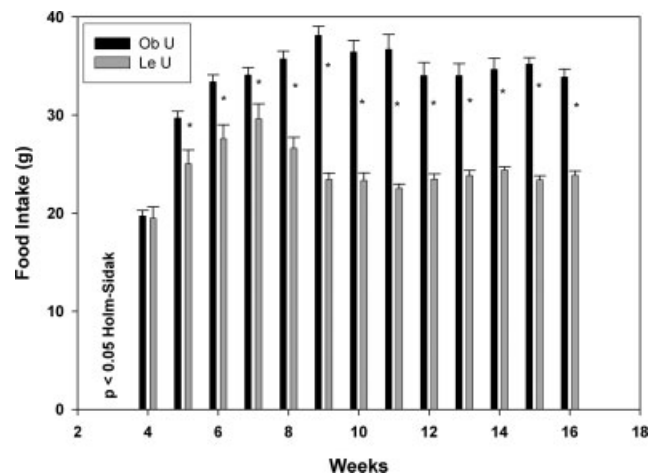


Fig. 2. Mean (\pm SEM) food intake of unrestricted Zucker rats over development. (*) $P < 0.05$.

Food intake

A two-way ANOVA showed significant main effects with respect to Strain ($F = 39.044$; $df = 1, 39$; $P < 0.001$; Fig. 2) and Age ($F = 272.048$; $df = 1, 39$; $P < 0.001$; Fig. 2). At 1 month, there were no differences between groups. By the fifth week, the rats began to show significant differences in food intake (Fig. 2) with the Le rats reaching a peak at 7 weeks and the Ob rats around 9 weeks. At 4 months Ob rats had higher food intake levels compared to Le ($t = 6.477$; $P < 0.05$; Fig. 2); Ob U rats consumed on average 30% more food than Le U rats. Finally, food intake levels were significantly higher at 4 compared to 1 month for both Ob ($t = 13.722$; $P < 0.05$; Fig. 2) and Le rats ($t = 9.604$; $P < 0.05$; Fig. 2).

Behavior assessment: Effects of apomorphine

This behavioral paradigm focuses on revealing behavioral differences in locomotor activity in response to APO administration rather than examining changes in general locomotor activity. APO is a nonselective DA agonist thought to bind with slightly higher affinity to D2-like than D1-like receptors (Dias et al., 2006; Jenner, 2003) and therefore this paradigm provides an additional measure of DA and D2R function in response to experimental treatment. For this reason the data were reported as the relative percent difference between saline and APO treated sessions for each group at 1 and 4 months.

A three-way ANOVA revealed significant differences in percent change of locomotor response to APO (Fig. 3) with respect to Strain ($F = 13.958$; $df = 1, 95$; $P < 0.001$) and Age ($F = 4.713$; $df = 1, 95$; $P = 0.033$) but not with respect to Diet. Subsequent multiple comparisons (Holm-Sidak) revealed several key differ-

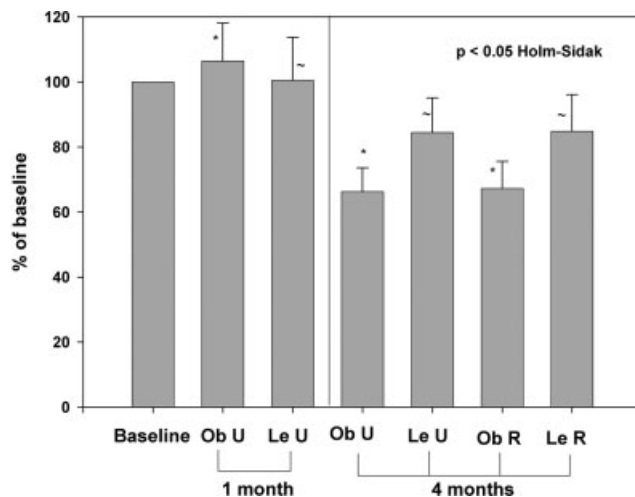


Fig. 3. Mean locomotor activity expressed as a percentage from baseline (SEM) at 1 and 4 months of age as assessed after intraperitoneal administration of 1 mg/kg APO. (*) Ob U and Ob R rats show decreased APO-induced locomotor activity between 1 and 4 months ($P < 0.05$), (~) Le U and Le R rats show decreased APO-induced locomotor activity between 1 and 4 months ($P < 0.05$).

ences. At 1 month, there were no differences between Ob and Le rats. At 4 months, APO caused a significant decrease in locomotor activity as compared to 1 month levels for all groups. Both unrestricted and restricted rats were equally affected, but the decline was more pronounced in Ob rats. Multiple comparison procedures (Holm-Sidak method) showed that percent changes in APO compared to saline-paired locomotor activity were lower in the Ob U ($t = 4.821$; $P < 0.05$), Ob R ($t = 4.712$; $P < 0.05$), Le U ($t = 3.467$; $P < 0.05$), and Le R ($t = 3.479$; $P < 0.05$) rats at 4 compared to 1 month of age and that this decline was more significant for the Ob (37%) than for the Le (16%) (see Fig. 3).

D2R autoradiography

Striatum

Brain sections were assessed with autoradiography for striatal D2R binding at 1 and 4 months (Fig. 4a). A three-way ANOVA showed significant differences with respect to Strain ($F = 45.341$; $df = 1, 63$; $P < 0.001$, Fig. 4b), Diet ($F = 38.915$; $df = 1, 63$; $P < 0.001$, Fig. 4b), and Age ($F = 41.748$; $df = 1, 63$; $P < 0.001$, Fig. 4b). Subsequent multiple pairwise comparison tests (Holm-Sidak method) were used to measure the differences of striatal D2R binding data between and within all groups. At 1 month, Le U rats had higher D2R binding levels as compared to Ob U rats ($t = 4.945$; $P < 0.05$). All four groups of rats at 4 months of age showed lower D2R binding levels than at 1 month. At 4 months, Le U rats had higher D2R binding levels than Ob U rats ($t = 4.719$, $P < 0.05$) whereas the differences between Le R and Ob R were not significant.

Also, Ob R rats had significantly higher D2R levels than Ob U ($t = 12.237$, $P < 0.05$) and Le R rats had higher D2R levels than Le U rats ($t = 12.315$, $P < 0.05$). Food restriction had a significant effect on age related D2R loss for both groups of rats; whereas D2R levels tended to decrease between 1 and 4 months in the unrestricted rats, this loss was attenuated in the restricted rats.

Cingulate cortex

A one-way ANOVA ($F = 7.389$; $df = 5, 39$; $P < 0.001$) was used to assess differences in D2R binding in the cingulate cortex (Fig. 4c). Multiple pairwise comparisons (Holm-Sidak) revealed no significant differences at 1 month of age. At 4 months we found significantly lower D2R binding in Ob U compared to 1 month old Ob U ($t = 4.148$; $p < 0.05$) and Le U ($t = 4.377$; $P > 0.05$) rats. Similarly, 4 month old Le U rats showed lower D2R binding compared to 1 month old Ob U ($t = 4.167$; $P < 0.05$) and Le U ($t = 4.410$; $P < 0.05$) rats. Unlike the unrestricted rats, D2R binding levels did not differ between 1 and 4 months in the restricted fed groups. At 4 months, Ob R and Le R rats showed greater D2R binding compared to Ob U ($t = 2.114$; $P < 0.05$) and Le U ($t = 2.874$; $P < 0.05$) rats, respectively. Le R rats also showed greater binding compared to Ob U rats ($t = 2.973$; $P < 0.05$).

Frontal cortex

A one-way ANOVA ($F = 5.608$; $df = 5, 39$; $P < 0.001$) was used to assess differences in D2R binding in the frontal cortex (Fig. 4c). Multiple pair-wise comparisons (Holm-Sidak) revealed no significant differences at 1 month of age. At 4 months we found significantly lower D2R binding in Ob U compared to 1 month old Ob U ($t = 3.876$; $P < 0.05$) and Le U ($t = 3.633$; $P < 0.05$) rats. Similarly, 4 month old Le U rats showed lower D2R binding compared to 1 month old Ob U ($t = 3.573$; $P < 0.05$) and Le U ($t = 3.310$; $P < 0.05$) rats. Unlike the unrestricted rats, D2R binding levels did not differ between 1 and 4 months in the restricted fed groups. At 4 months, Le R rats showed greater D2R binding compared to Le U ($t = 3.078$; $P < 0.05$) and Ob U ($t = 3.403$; $P < 0.05$). Finally, at 4 months Le U rats showed greater D2R binding compared to Ob U ($t = 2.044$; $P < 0.05$).

Weight vs. D2R binding levels in striatum

The plot showing the relationship between striatal autoradiography D2R binding and weight was fitted with a linear regression model (Fig. 5). The regression model showed a significant relationship between D2R and weight across groups ($r = -0.90$; $P < 0.0001$; Fig. 5).

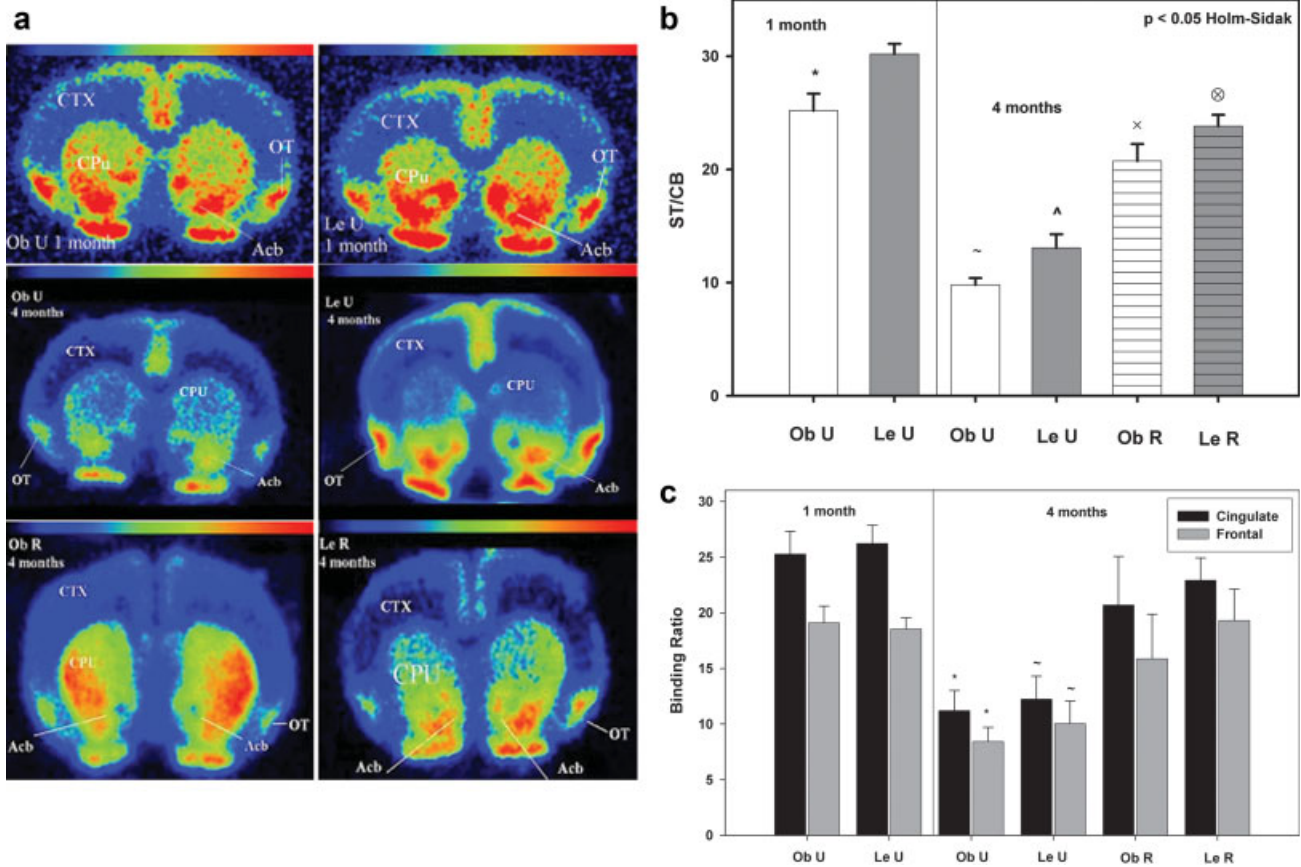


Fig. 4. (a) Digital autoradiograms of [^3H] spiperone autoradiography at 1 month and 4 months in Ob and Le Zucker rats. CTX, cerebral cortex; Acb, nucleus accumbens, OT, olfactory tubercle. (b) Mean (+SEM) [^3H] spiperone binding ratio (receptor rich/receptor poor) in Ob and Le Zucker rats at 1 and 4 months in the striatum. (*) 1 month old Ob U rats show decreased D2R binding compared to 1 month old Le U rats ($P < 0.05$). (~) 4 month old Ob U rats show decreased D2R binding compared to 1 month old Ob U and 4 month old Ob R rats ($P < 0.05$), (^) Le U show decreased D2R binding compared to 1 month old Le U and 4 month old Le R rats

($P < 0.05$). (x) Ob R rats show decreased D2R binding compared to 1 month old Ob U rats ($P < 0.05$) (⊗) Le R rats show decreased D2R binding compared to 1 month old Le U rats ($P < 0.05$). (c) Mean (+SEM) [^3H] spiperone binding ratio (receptor rich/receptor poor) in Ob and Le Zucker rats at 1 and 4 months in the cingulate and frontal cortices. (*) 4 month old Ob U rats show decreased D2R binding compared to 1 month old Ob U and 4 month old Ob R rats (~) 4 month old Le U rats show decreased D2R binding compared to 1 month old Le U and 4 month old Le R rats.

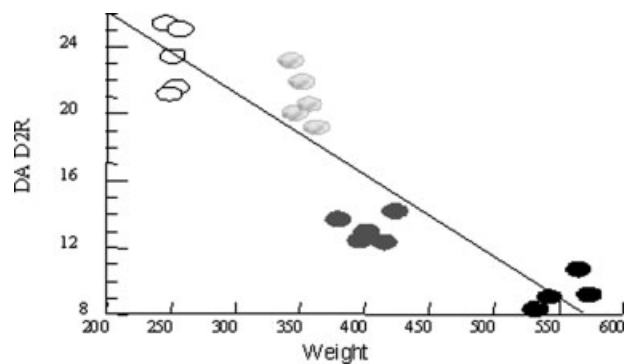


Fig. 5. Regression plot for the relationship between weight (grams) and D2R autoradiography binding potential levels (ST/CB) for the lean restricted (white circles), the obese restricted (light gray circles), lean unrestricted (dark gray circles) and obese unrestricted (black circles). The regression is highly significant ($r = -0.90$, $P < 0.0001$).

Plasma insulin and leptin

Insulin

A two-way ANOVA revealed significant main effects (Fig. 6a) with respect to Strain ($F = 279.17$; $df = 1, 39$; $P < 0.001$), Diet ($F = 81.64$; $df = 1, 39$; $P < 0.001$) and their interaction ($F = 96.48$; $df = 1, 39$; $P < 0.001$). Subsequent pairwise multiple comparisons revealed significantly greater plasma insulin concentrations in Ob U compared to Ob R ($t = 13.33$; $P < 0.001$) and Le U ($t = 18.76$; $P < 0.001$). Within the two restricted groups, Ob R had higher plasma insulin concentrations than Le R rats ($t = 4.87$; $P < 0.001$) but there was no significant difference in insulin concentration between Le U and Le R rats ($t = 0.56$; $P = 0.58$).

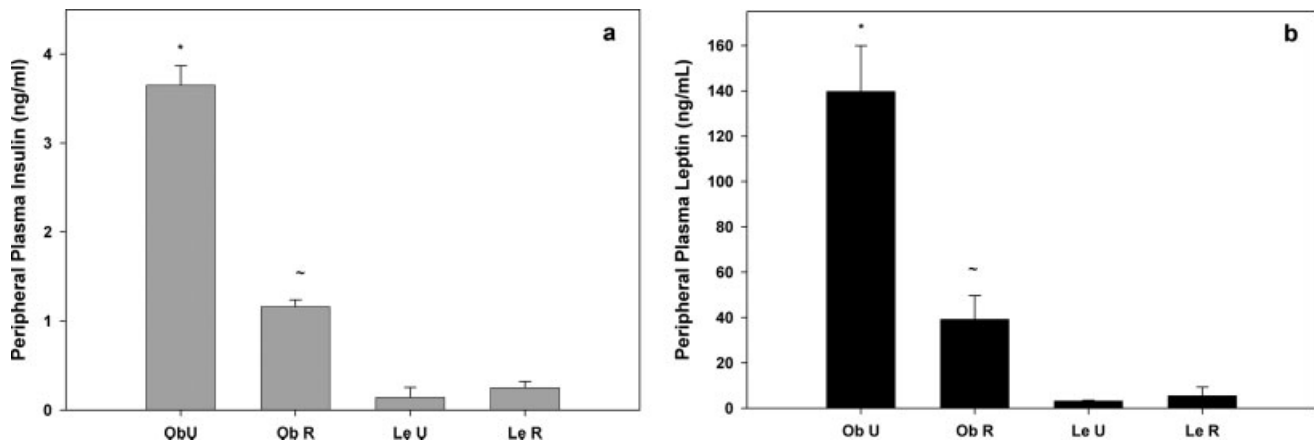


Fig. 6. (a) Mean (+SEM) Fasted (24 h) plasma insulin concentrations in 4 month old Obese and Lean Zucker rats (*) Ob U rats show greater insulin levels compared to Ob R, Le U and Le R rats ($P < 0.05$) (~) Ob R rats show greater insulin levels compared to Le U and Le R rats ($P < 0.05$) (b) Mean (+SEM) Fasted (24 h) plasma

leptin concentrations in 4 month old Obese and Lean Zucker rats (*) Ob U rats show greater leptin levels compared to Ob R, Le U and Le R rats ($P < 0.05$). (~) Ob R rats show greater leptin levels compared to Le U and Le R rats ($P < 0.05$).

Leptin

A two-way ANOVA revealed significant main effects (Fig. 6b) with respect to Strain ($F = 284.26$; $df = 1, 39$; $P < 0.001$), Diet ($F = 77.49$; $df = 1, 39$; $P < 0.001$), and their interaction ($F = 87.67$; $df = 1, 39$; $P < 0.001$). Subsequent pairwise multiple comparisons revealed significantly greater plasma leptin concentrations in Ob U compared to Ob R ($t = 9.47$; $P < 0.001$) and Le U ($t = 17.24$; $P < 0.001$). Within the two restricted groups, Ob R had higher plasma leptin concentrations than Le R rats ($t = 3.36$; $P < 0.001$) but there was no significant difference in leptin concentrations between Le U and Le R rats ($t = 0.64$; $P = 0.69$).

D2R μ PET imaging

We were unable to utilize a three-way ANOVA design due to differences in the number of data points between the different levels of the three factors (Strain, Diet and Time) and therefore we chose a two factor model which does not require equal sample sizes. The reason for the differences in sample sizes stems from the fact that the data analyzed consisted of individual ROI values and in most cases the ROI values obtained differed from animal to animal based on the integrity of the μ PET images. Measures were examined at two time points for each group of rats: 1 and 4 months (Figs. 7a and 7b). A two-way ANOVA (Fig. 7b) showed a main effect for Age ($F = 199.086$; $df = 1, 359$; $P < 0.001$) and Group ($F = 18.481$; $df = 3, 359$; $P < 0.001$) but there was no interaction effect. Since there was no interaction effect, the subsequent pairwise multiple comparisons (Holm-Sidak method) assessed a generalized comparison for all data at 1 and 4 months. This showed significantly greater D2R

availability at 1 versus 4 months ($t = 14.762$; $P < 0.001$) for all groups. To assess individual group differences at 1 versus 4 months of age a one-way ANOVA was conducted ($F = 48.250$; $df = 5, 359$; $P < 0.001$; Fig. 7b) and this yielded greater D2R availability in 1 month old Ob U rats (Fig. 7b) compared to 4 month old Ob U ($t = 8.503$; $P < 0.001$), Le U rats ($t = 13.534$; $P < 0.001$), Ob R ($t = 7.003$; $P < 0.001$), and Le R ($t = 7.121$; $P < 0.001$). The one-way ANOVA showed greater D2R availability in 1 month old Le U rats (Fig. 7b) compared to 4 month old Le U ($t = 11.851$; $P < 0.001$), Le R ($t = 5.752$; $P < 0.001$), Ob U ($t = 7.258$; $P < 0.001$), and Ob R ($t = 5.790$; $P < 0.001$). At 4 months of age, Ob U rats showed significantly greater D2R binding than Le U rats ($t = 4.593$; $P < 0.001$) and showed a trend towards lower D2R binding values than Ob R rats ($t = 1.861$; $P = 0.06$). Also, Le R had significantly greater D2R binding than Le U rats ($t = 7.618$; $P < 0.001$). Finally, 4 month old Ob R rats had greater D2R availability than 4 month old Le U rats ($t = 6.702$; $P < 0.001$; Fig. 7b).

Comparison of nonfasted (NF) versus the 24 h fasted (F) conditions in the 4 month old Ob and Le ad-lib fed animals (two-way ANOVA) showed a main effect for Group ($F = 34.77$; $df = 3, 335$; $P < 0.001$) and fed or fasting State ($F = 14.65$; $df = 1, 335$; $P < 0.001$) (Fig. 7c). The interaction effect between the above two factors was not significant and therefore the subsequent pairwise multiple comparisons (Holm-Sidak method) compared all data between the NF and F state. This showed significantly greater D2R availability in the NF versus F states ($t = 3.99$; $P < 0.001$; Fig. 7c) for all the groups. To assess individual group differences in the NF versus F state, a one-way ANOVA was conducted ($F = 20.85$; $df = 5, 335$; $P < 0.001$).

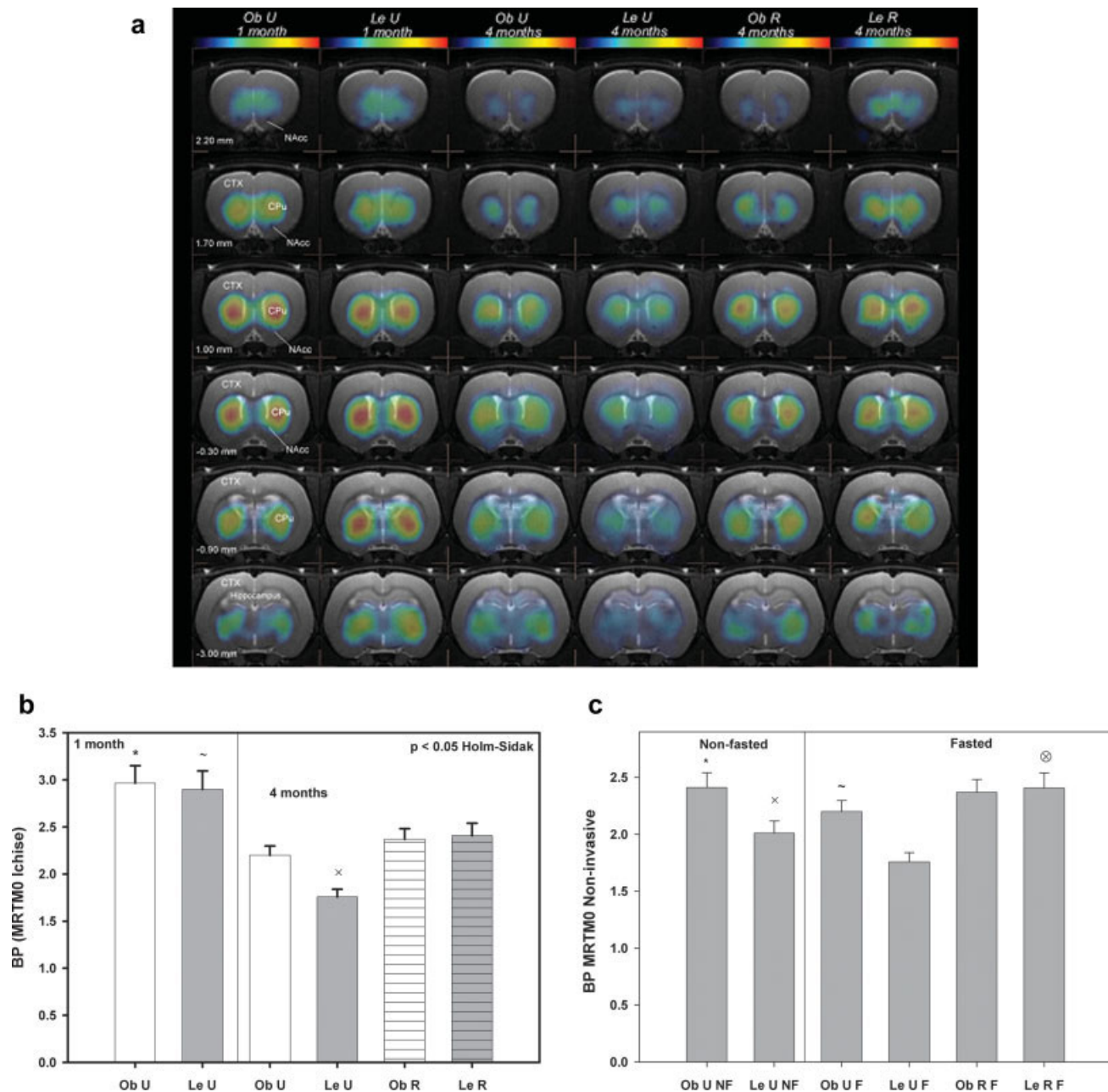


Fig. 7. (a) [^{11}C] raclopride μPET imaging of Ob and Le Zucker rats at 1 and 4 months of age. (b) Mean (+ SEM) [^{11}C] raclopride μPET MRTM0 Ichise Binding Potential (BP; k_3/k_4+1) of Ob and Le Zucker rats at 1 and 4 months of age. (*) 1 month old Ob U rats show greater D2R availability compared to 4 month old Ob U and Ob R rats ($P < 0.05$). (~) 1 month old Le U rats show greater D2R binding availability compared to 4 month old Le U and Le R rats ($P < 0.05$). (x) 4 month old Le U rats show lower D2R availability compared to 4 month old Le R rats ($P < 0.05$). (c) Mean (+SEM)

MRTM0 [C-11] raclopride binding potential in fasted (F) and non-fasted (NF) 4 month old Obese and Lean Zucker rats. (*) NF Ob U rats show greater D2R availability compared to NF Le U and F Ob U rats ($P < 0.05$). (~) F Ob U rats show greater D2R availability compared to F Le U rats ($P < 0.05$). (x) NF Le U rats showed greater D2R availability compared to F Le U rats ($P < 0.05$). (x) F Le R rats show greater D2R availability compared to F Le U rats ($P < 0.05$).

0.001; Fig. 7c) and this yielded greater D2R availability in the NF Ob U rats compared to F Ob U ($t = 2.47$; $P < 0.01$); NF Le U ($t = 4.67$; $P < 0.001$) and F Le U rats ($t = 7.61$; $P < 0.001$). F Ob U rats showed greater D2R availability than F Le U rats as well ($t = 5.15$; $P < 0.001$). Also, the F Ob R rats had sig-

nificantly greater D2R binding availability than F Le U ($t = 7.51$; $P < 0.001$) and NF Le U ($t = 4.41$; $P < 0.001$).

The one-way ANOVA also showed greater D2R availability in NF Le U vs. F Le U rats ($t = 2.94$; $P < 0.01$). Finally, F Le R rats had higher D2R binding

than F Le U ($t = 8.54$; $P < 0.001$), NF Le U ($t = 5.22$; $P < 0.001$) and F Ob U ($t = 2.73$; $P < 0.01$).

DISCUSSION

Food intake and weight gain

At 1 month we show no significant difference in weight and food intake between Ob and Le rats whereas at 4 month the differences were very apparent; Ob rats weighed 39% more and consumed 42% more than their lean counterparts. As expected food restriction reduced weight at 4 months in Ob R (compared to Ob U; 55% difference) and Le R rats (compared to Le U; 51% difference), which was consistent with previous results (Meguid et al., 2000). These findings corroborate the regulatory role of leptin in maintaining food consumption regardless of food availability.

Apomorphine-paired locomotor activity

At 1 month, we did not observe differences in the percent change of locomotor response to APO administration in Ob and Le unrestricted rats, suggesting that Ob and Le rats at this age may be characterized by similar DA and D2R profiles. The percent decline in APO-paired locomotor activity between 1 and 4 months was greater for Ob U (38% decrease) and Ob R (38% decrease) than for Le U (16% decrease) and Le R rats (16% decrease). The difference in percent changes in locomotor activity in response to APO between the Ob and Le strains (38% vs. 16%) regardless of food restriction suggests an involvement of LepR in regulating the effects of a DA agonist (apomorphine) on locomotor activity. The decreased sensitivity to the locomotor effects of apomorphine in the LepR deficient rats (regardless of their weight) suggests that leptin regulation of DA receptors is functionally significant.

D2R characterization using μ PET and β -imager ARG

μ PET and ARG yielded different results in the characterization of the effects of strain and food restriction on D2R. This is likely to reflect differences between the two procedures. Specifically, μ PET is conducted under in-vivo conditions and therefore D2R binding is subject to several additional factors. One major factor is competition of endogenous DA with raclopride. Raclopride is in fact highly displaceable by DA (Morris and Yoder, 2007) and therefore any increases in endogenous DA would likely show up as decreases in D2R binding and vice-versa. Similarly, behavioral status (fed vs. fasted) (Miklya et al., 2003) and anesthesia may affect DA concentration and thus could influence raclopride's binding levels. While, to our knowledge there is no evidence that ketamine,

(the specific anesthetic used in this study), binds to the D2R in-vivo; it has previously been shown that surgical concentrations of isoflurane, halothane, ethanol and ketamine inhibit high affinity states of the D2R as well as other G-protein linked receptors in-vitro (Kapur and Seeman, 2002; Seeman and Kapur, 2003). In the present study, all animals were administered ketamine during the scanning period and therefore any effects on the D2R would apply to all groups. On the other hand for ARG the influence of endogenous DA was minimized by pre-washing the slides with specific buffers. Also spiperone is much less sensitive to displacement by DA than raclopride (Morris and Yoder, 2007). Therefore we have used ARG which is insensitive to competition to endogenous DA and provides a much better spatial resolution as the measure to evaluate D2 receptor levels. On the other hand we have used the discrepancies between the ARG and the μ PET studies to infer potential changes in endogenous DA that could influence the binding of D2R for raclopride but not for spiperone. However, because raclopride but not spiperone also binds to D3 receptors in striatum the differences could also reflect changes in D3 receptors expression.

D2R as a function of strain

At 1 month of age ARG revealed significantly higher ($\sim 20\%$) D2R binding in Le U compared to Ob U rats. This differs from previous findings showing no differences in D2R density (Bmax) nor affinity (Kd) between Ob and Le Zucker rats (Hamdi et al., 1992) at 1 month of age. We also show a 56% decline in striatal D2R in Le U rats and a 60% decline in Ob U between 1 and 4 months of age. This also differs from a prior study that reported a 51% increase in D2R levels in Le U rats between 1 and 6 months, while D2R levels remained unchanged for Ob U rats. The above discrepancies may be explained both by differences in sensitivity (500% higher for autoradiography using a β -imager compared to film) (Langlois et al., 2001) as well as differences in the D2R radioligands used. Specifically, in the Hamdi 1992 article, [3 H]nemonapride and not [3 H]spiperone was used for D2R binding. These two ligands differ significantly in the labeling of D2R (Vivo et al., 2006). Also, it appears that Hamdi did not utilize a prewash in his binding protocol to remove D2R bound DA and therefore binding in that experiment may have been done in the presence of DA while in our study it was not.

Our findings of low D2R levels in Ob U rats corroborate the findings from recent clinical imaging data showing that obese individuals have reduced D2R binding levels compared to lean controls (Wang et al., 2001, 2004). Note that human obesity resembles the unrestricted food condition in the animal model except for the fact that while the Ob Zucker rats have

LepR inactivation most obese subjects do not. Similar to the human data (inverse correlation between D2R striatal levels and the BMI in the obese subjects), we also documented an inverse correlation in the Ob rats between D2R levels and their weight (Fig. 5). Since we found more D2R binding in Le U relative to Ob U rats, we may assume that Ob rats experience a greater decrease of D2R expression as they age. The fact that age related D2R decline was attenuated with food restriction may suggest that the greater D2R loss with age in the Ob U reflects increased food intake and weight gain (Wang et al., 2001,2004). The mechanisms that mediate this downregulation of D2R expression are unclear but are unlikely to reflect the effects of leptin since the effects of food restriction were observed in the Ob R, which has no leptin receptor function. It's also unlikely to reflect insulin since food restriction also decreased the age-related decreases in D2R in Le R rats when compared to Le U even though both their insulin and leptin levels did not differ from one another. These findings are consistent with prior studies showing that food restriction alters the effects of aging (Ingram et al., 2001) on motor activity and reward (Carr et al., 2003), and thus an attenuation of age-induced loss of D2R could underlie the benefits that food restriction has on these behaviors.

In contrast to the lower D2R in Ob than in Le rats revealed by ARG, μ PET revealed the opposite; that is greater D2R availability in Ob than Le. This discrepancy could be explained if Ob rats had lower DA release than Le rats or alternatively had a higher expression of D3 receptors. Indeed there is evidence from microdialysis studies of lower DA levels in the ventral striatum of leptin-deficient obese mice (ob/ob) when compared to controls (Fulton et al., 2006) and of attenuated striatal dopaminergic responses to insulin in obese Zucker rats (Orosco et al., 1992). It has been shown that Obese Zucker rats have high insulin levels (Zucker and Antoniades, 1972) (Stern et al., 1972), a finding that we observe as well and insulin has been associated with increased DAT mRNA (Figlewicz et al., 1998) as well as with increased sensitivity of DAT for DA uptake (Owens et al., 2005). So, the high DAT mRNA and the more efficient DA uptake by DAT associated with hyperinsulinemia in Ob Zucker rats could translate to lower DA signaling. Indeed decreases in both striatal DA and DOPAC levels have been reported in Ob Zucker rats (Shimizu et al., 1991).

It has been suggested that weak DAergic signaling in hypothalamus, which is involved with satiety, could result in greater food consumption to bring about satiety (Orosco et al., 1995). However it is unclear how decreased striatal DAergic signaling in Ob rats relates to their increased food intake. Since the mesolimbic and striatonigral DA pathways that target the

ventral and the dorsal striatum, respectively, have been implicated in reward (and prediction of reward) and motivation; an alternative suggestion is that deficiency in these pathways may lead to pathological eating as a means to compensate for low motivation, reward, or saliency. Also microdialysis studies are required to determine if indeed Ob rats have reduced baseline DA extracellular level in striatum. Similarly ARG studies to measure D3 receptors in striatum would enable to determine if there are increases in the levels of receptors in the striatum of the Zucker Ob rats.

Effects of acute food restriction in D2R availability as measured with μ PET in ad-lib animals

Here we show that in unrestricted animals 24 h fasting was associated with a decrease in D2R availability when compared with non fasting conditions. We interpret this to indicate that 24 h of fasting results in increases in DA release in striatum. Since this effect was shown both in the Ob and the Le rats we postulate that is not related to leptin effects. This is consistent with prior studies showing that fasting increases DA release in the striatum in ad-lib fed Wistar Rats (Miklya et al., 2003). Moreover, 24 h fasting has been shown to decrease mRNA levels for the DA transporter (Patterson et al., 1998), which is the main mechanism for regulating extracellular DA concentration (Gainetdinov et al., 1999) and thus would be expected to increase extracellular DA levels. This would explain the higher D2R binding potential that we observed with raclopride in the unrestricted (Ob U and Le U) than in the fasted condition.

In this study we chose a 24 h fasting period because restricted rats were fed once every 24 h and usually consumed all of the food within an hour of being fed. In this way, it is as if they were consistently fasted for a period of ~24 h. Fasting both the restricted and ad-libitum fed rats for this amount of time provides a basis for the direct comparison of changes in D2R binding availability in the four experimental groups during a fasting period. The caveat here is that the restricted rats are conditioned to the food restriction while the ad-libitum fed rats are not. This raises the question of how D2R binding availability of a rodent who is on a chronically restricted feeding regiment differs from that of a rodent who is not when both are in a state of negative energy balance that is brought upon by reduced food intake.

DA differences between chronic food restriction in Ob and Le Zucker rats and acute (24 h fasting) food restriction

Restricted Le rats had greater D2R availability when tested with μ PET than their unrestricted counterparts.

The Ob R also tended to have higher availability than Ob U though these differences were not significant. This was consistent with the ARG findings which also showed greater D2R in restricted than unrestricted rats suggesting that this is driven mostly by differences in D2R levels than by major changes in DA release. However because [^{11}C] raclopride is sensitive to endogenous DA we can not rule out the possibility of changes in DA between chronically restricted and unrestricted rats. Indeed, "subchronic" food restriction (1 week) has been shown to lead to a decrease in extracellular DA in the NAc (Pothos et al., 1995a,b). Also chronic food deprivation and diet-induced obesity, two conditions that are characterized by differences in feeding, have both been associated with decreases in extracellular DA levels in the ventral striatum (Pothos et al., 1998). Similarly, obese and lean female ad-libitum fed Zucker rats did not differ in striatal DA metabolites at 5 weeks of age but by 16 weeks the obese had significantly lower DA metabolites in the striatum compared to lean rats (Orosco et al., 1986). The above studies point to a low basal release of DA in chronically food restricted and obese rats and suggest that the low basal DA levels may be due in part to increased storage of DA in synaptic vesicles.

Limitations

1. Here we interpret the differences between μPET and ARG as reflecting the competition of raclopride with endogenous DA and the binding of spiperone as mostly reflecting D2R levels. However we also recognize that other properties of the radioligands used for μPET ([^{11}C] raclopride) and for ARG ([^3H] spiperone), may have contributed to the differences in results. Raclopride binds both to D2 and D3 receptors whereas spiperone binds to D2 and D4 receptors. This may be relevant since the D3R signaling appears to be involved in the regulation of body weight (McQuade et al., 2004). The two tracers may also bind to D2R differentially (raclopride binds to cells in the membrane while spiperone may penetrate the cell membrane and bind to internalized receptors as well) (Laruelle, 2000).
2. The values for D2R binding availability that we report reflect the average for the entire striatum (Fig. 7a) as well as partial volume and spillover effects which are a limitation of μPET (Laforest et al., 2005; Thanos et al., 2002).
3. Neither PET nor ARG allow us to differentiate between pre and postsynaptic D2R.
4. The relationship between the observed effects on D2R availability as inferred from μPET and the actual release of DA remains speculative and further validation studies are required for the proper characterization of this methodology. This is fur-

ther confounded by the inability to distinguish between tonic vs. phasic release of DA in response to food consumption in the two strains. Nevertheless, it provides a longitudinal approach to the study of DA system function without having to deal with complicated issues like tissue integrity and survival which are both significant limitations of other approaches (i.e., microdialysis, dissections).

5. We can not rule out the possibility that the differences in D2R are independent of leptin effects and may reflect adaptation responses (peripheral and central) to the leptin receptor mutation in these rats.

SUMMARY

Here we show using ARG lower D2R levels in the CNS of Ob than in Le rats and in food unrestricted than food restricted conditions indicating that both genotype as well as food availability affect D2R levels. We also show attenuation by food restriction of the D2R decline with aging that was most accentuated in Ob than in Le rats. This finding provides evidence for a role of leptin in modulating adaptation responses of the DA system with respect to chronic food restriction. In as much as food restriction attenuates the D2R losses associated with aging this may underlie the salutary effects that food restriction has on locomotor activity and on sensitivity to reward in aged animals. Finally by assessing binding with [^{11}C] raclopride, which is sensitive to competition with endogenous DA, we provide indirect preliminary evidence that extracellular DA may be decreased in Ob when compared with Le rats and that acute fasting (24 h) enhances DA extracellular levels.

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